

**INTER- KINGDOM RECOGNITION OF NOREPINEPHRINE BY *E. COLI*:
IDENTIFICATION OF THE RECEPTORS INVOLVED IN CHEMOTAXIS**

A Thesis

by

DAE NYUN KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Arul Jayaraman
Committee Members,	Zhilei Chen
	Robert C. Alaniz
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ABSTRACT

Inter-kingdom Recognition of Norepinephrine by *E. Coli*: Identification of the Receptors

Involved in Chemotaxis. (August 2012)

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Chair of Advisory Committee: Dr. Arul Jayaraman

There are approximately 10^{14} bacteria belonging to nearly 1000 different species in the human gastrointestinal (GI) tract that co-exist with host cells. Within the GI tract, signaling molecules secreted by both eukaryotic and prokaryotic cells are abundant. Recent studies have shown that both bacteria and human cells recognize and respond to the signals from each other, presumably to gain a competitive advantage. The cross-recognition of signals is known as Inter-kingdom (IK) signaling and this phenomenon is considered to be important in the onset of infections in the GI tract. Of the eukaryotic signaling molecules present in the GI tract, the neuroendocrine hormone norepinephrine (NE) is considered to be important in the context of infections as NE is produced at very high concentration in the intestine under post traumatic stress, is known to increase bacterial virulence and infection, and has also been shown to be a potent chemoattractant for GI tract pathogens such as enterohemorrhagic *E. coli* (EHEC). The focus of this study is on elucidating the mechanisms underlying the recognition and chemotaxis of bacteria towards NE.

While chemotaxis has been typically investigated in the context of bacteria moving towards a metabolizable source (e.g., amino acids), chemotaxis is potentially important in the onset of infections in the human GI tract. In this study we use a microfluidic plug

assay to investigate the receptor and mechanism utilized by a model bacterium *Escherichia coli* in its chemotactic response to NE. A series mutant of *E. coli* RP437 strains of knockouts for four MCP-encoding genes was used in this study. The results from the microfluidic plug assay were then confirmed quantitatively by capillary assay.

We have shown that Tsr receptor is necessary for chemotaxis of NE for *E. coli* RP437, and attraction of *E. coli* towards NE may require an additional receptor. Results from the priming experiments suggest that exposure to NE may result in the de novo expression of co-receptor(s) that are crucial to chemotaxis towards NE. The requirement for high cell density also suggests the possibility that NE per se may not be an attractant for *E. coli*, but could be a precursor that is modified into a chemoattractant by cells. These results are expected to further our understanding of bacterial chemotaxis and its role in bacterial colonization and infection of the human GI tract.

DEDICATION

To my wife and my yet to be born child
who completes me

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I would like to thank my committee chair Dr. Arul Jayaraman for inspiring me, and troubleshooting my experiments. I thank him for being so kind and understanding even at my worst times. Without him, this thesis would not have seen the light of day.

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Most importantly, I thank my family. I especially like to mention my grandfather, who is not with us anymore, for his unconditional love and for directing me to the right

road. I would not be here without the wisdom of my grandfather. My parents have always believed in and supported my choices indubitably. I thank my father-in-law and mother-in-law for trusting me with their daughter.

Lastly, I dedicate this thesis to my loving wife and yet to be born child. My wife, mother of my yet-to-be born baby, Seahyun has been the most understanding person and is the best companion for life that a man can ask for. I am sorry for dragging you out here to the land of boredom. I hope I can repay all the debts to you in this lifetime. I love you.

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CHAPTER I

INTRODUCTION

1.1 Background

The human GI tract is a nutrient-rich environment where approximately 100 trillion (10^{14}) microbes from up to 1000 distinct bacterial species exist, which is approximately 10-times greater than the number of cells in the human host (1). Therefore, the gut microbiome is one of the most complex microbial ecosystems, in which a diverse population of bacteria co-exists with human cells (2). The gut microbiome contains symbionts (or probiotics) that have known health-promoting functions, commensals, that are permanent residents that provide no benefit or cause no harm to the host (3), and pathobionts (or pathogens) that have the potential to induce pathological changes in the human host (4). Symbiotic gut bacteria supply essential nutrients, metabolize compounds in the food, defend against colonization by foodborne pathogens and by opportunistic-pathogens, and contribute to the development of overall health of humans (3).

Bacterial chemotaxis is one of the simplest and best-understood behaviors at the molecular level. It is the biasing of movement towards regions that contain higher concentrations of beneficial, or lower concentrations of toxic, chemicals. The in vivo

This dissertation follows the style of *Proceedings of the National Academy of Sciences of the United States of America*.

relevance of bacterial chemotaxis is self-evident. Chemotaxis towards epithelial cells in the GI tract in response to small molecules has been shown to be a crucial first step in infection (5). Because the migration to host cell surfaces is the crucial first step for many pathogens, chemoeffectors in the microenvironment where infections occur have been proposed to be determinants of infection (6). For example, *Helicobacter pylori* has been described to use chemotaxis for migrating to gastric epithelial cell surfaces (7, 8). Although recent reports that chemotaxis affects the virulence of pathogenic bacteria (7-9) and the observation that molecules such as hormones present in the GI tract influence different phenotypes associated with virulence and chemotaxis (5, 10, 11), there is very little information on how the chemotaxis response to specific molecules leads to establishment of infections in living organisms.

Several signaling molecules within the GI tract have been shown to affect chemotaxis and virulence (5, 10, 11). Of these hormones such as norepinephrine, dopamine, and serotonin that are produced locally in the GI tract via the enteric nervous system are the best studied (12-15). NE is a major neurotransmitter of the sympathetic nervous system that enervates the GI tract, and L-dopa, which is a precursor in the NE biosynthesis pathway, is a component of food, such as bananas (14, 16). With more than 100 million nerve endings enervating the smooth muscle that underlies the intestinal epithelium throughout the GI tract (12, 13), local concentrations of NE in the GI tract lumen can be extremely high. The NE concentration becomes elevated in stress conditions (17) and that susceptibility to infections increases during these states. About 50% of all the NE

found in the body is synthesized in the GI tract (12, 18). Therefore, it is likely that NE can spill-over into the lumen, where it could be sensed by different pathogens.

NE has been reported to be a chemoattractant for enterohemorrhagic *E. coli* (10). However, the receptor(s) involved in NE sensing have not yet been identified. This work focuses on identifying the receptor(s) involved in the chemo-sensing of NE in *E. coli*. A non-pathogenic lab strain *E. coli* RP437 will be used as a model strain for investigating the chemotaxis of EHEC towards GI tract molecules, since both non-pathogenic *E. coli* and EHEC possess the same five chemoreceptors Tar, Tsr, Tap, Trg, and Aer with a high degree of gene and protein sequence homology (19). Moreover, *E. coli* RP437 is well characterized, has been extensively used for chemotaxis studies, and a mutant library with different combinations of deleted chemoreceptors is also available (20).

1.2 Motivation

Infections caused by foodborne pathogens affect millions of people and kill thousands in the United States alone. The Center for Disease Control (21) estimates for 2011 show that food-borne infections affect approximately 47.8 million Americans every year, with 128,000 hospitalizations and 3,000 deaths (21). Foodborne pathogens such as *Salmonella* spp., EHEC, enteropathogenic *E. coli* (EPEC), *Campylobacter* spp., and *Staphylococcus aureus* are the most common causes of enteric infections. Enteric infections are characterized by diarrhea, abdominal discomfort, nausea and vomiting, and anorexia, and sometimes lead to infection in the systemic circulation and death. In

United States, estimation of \$152 billion/year, including the medical costs (hospital services, physician services, and drugs), quality-of-life losses (deaths, pain, suffering, and functional disability), and costs to others in society (e.g. costs to insurance companies that pay medical expenses) is associated with foodborne illness alone (22). Some enteric infections, such as the ones caused by EHEC, cannot be treated by antibiotics because of the possibility of aggravating the infection due to enhanced release of shiga toxins that are critical for the infection (23); this requires doctors to employ alternate treatment approaches such as fluid and electrolyte administration to limit the severity of symptoms (24, 25).

1.3 Research importance, objectives, and novelty

Because the host cells and bacteria in the GI tract are in close proximity, a pathogen entering the GI tract are exposed to molecules produced by the host mucosal cells as well as by the commensal bacteria that reside in the GI tract. It has been proposed that the pathogens utilize these molecules to identify the favorability of the microenvironment for colonization and further to infection. These molecules include those used in bacterial quorum sensing or cell-cell communication and metabolites produced during normal bacterial growth. Eukaryotic signals produced *in situ* in the intestine of the host include hormones such as norepinephrine (NE), epinephrine, dopamine, and serotonin (26) and small molecules such as adenosine (27). Importantly, it has been shown that molecules produced by bacteria in the GI tract are recognized by

host cells and vice versa (26). Therefore, the recognition of the produced molecules by cells of different kingdoms and the high abundance of these molecules are thought to be a major contributor to the initiation and development of pathogen infections in the GI tract. However, previous studies have typically focused on either the response of pathogenic bacteria to bacterial signals, or on the role of hormones in the context of host GI tract physiology. Importantly, the role of these hormones in chemotaxis and initiating infections are not fully understood. Specifically, the mechanism(s) underlying chemotaxis in the GI tract are not fully known, and evidence that firmly establishes the role of chemotaxis in colonization is limited. Thus the proposed experiments are novel because bacterial chemotaxis has not been studied from the view point of IK signaling and this work will be the first time providing a molecular basis for this IK interaction.

CHAPTER II

LITERATURE REVIEW

2.1 Bacterial Chemotaxis

2.1.1 *Escherichia coli* chemotaxis

Chemotaxis, or the movement toward or away from chemicals, is a universal attribute of motile cells and organisms (28). *E. coli* cells swim toward amino acids (e.g., serine, aspartic acid), sugars (maltose, ribose, galactose, glucose), dipeptides, pyrimidines and electron acceptors (oxygen, nitrate, fumarate) (29-31). Cells also swim away from potentially harmful chemicals, such as alcohols and fatty acids, but repellent responses have not been as extensively studied (32). In the absence of any stimulating chemical gradient, *E. coli* swims in a random walk pattern produced by alternating episodes of counter-clockwise (CCW) and clockwise (CW) flagellar rotation (33). In an attractant or repellent gradient, the cells monitor chemoeffector concentration changes as they move and use that information to control the probability of the next tumbling event (34). These flagellar responses extend runs that take the cells in favorable directions (toward attractants and away from repellents), resulting in net movement toward preferred environments. *E. coli* senses chemoeffector gradients in a temporal fashion by comparing the current concentrations to those encountered over the past few seconds of travel. Out of the five chemoreceptors in *E. coli*, four (Tsr, Tar, Tap, and Trg) are transmembrane receptors. These methyl-accepting chemotaxis proteins (MCPs) have

periplasmic ligand binding sites and conserved cytoplasmic signaling domains (34) (**Fig. 2.1**) and record the cell's recent chemical past (ligand concentration) in the form of reversible methylation of specific glutamic acid residues in the cytoplasmic signaling domain of the chemoreceptors (34). Whenever the ligand bound to the periplasmic domain of the MCP's dissociates from the receptor, the flagellar motor response stops until a new ligand binds to the MCP (34). A fifth MCP-like protein, Aer, mediates aerotactic responses by monitoring redox changes in the electron transport chain (34). Aer undergoes sensory adaptation through a poorly understood, methylation-independent mechanism. The five MCP-family receptors in *E. coli* utilize a common set of cytoplasmic signaling proteins to control flagellar rotation and sensory adaptation (**Fig. 2.1**) (34). Receptor CheW and sensor kinase CheA generate receptor signals in the form of phosphoryl groups to CheY and CheZ which control motor responses in response to chemical stimuli; CheR and CheB regulate MCP methylation state (34).

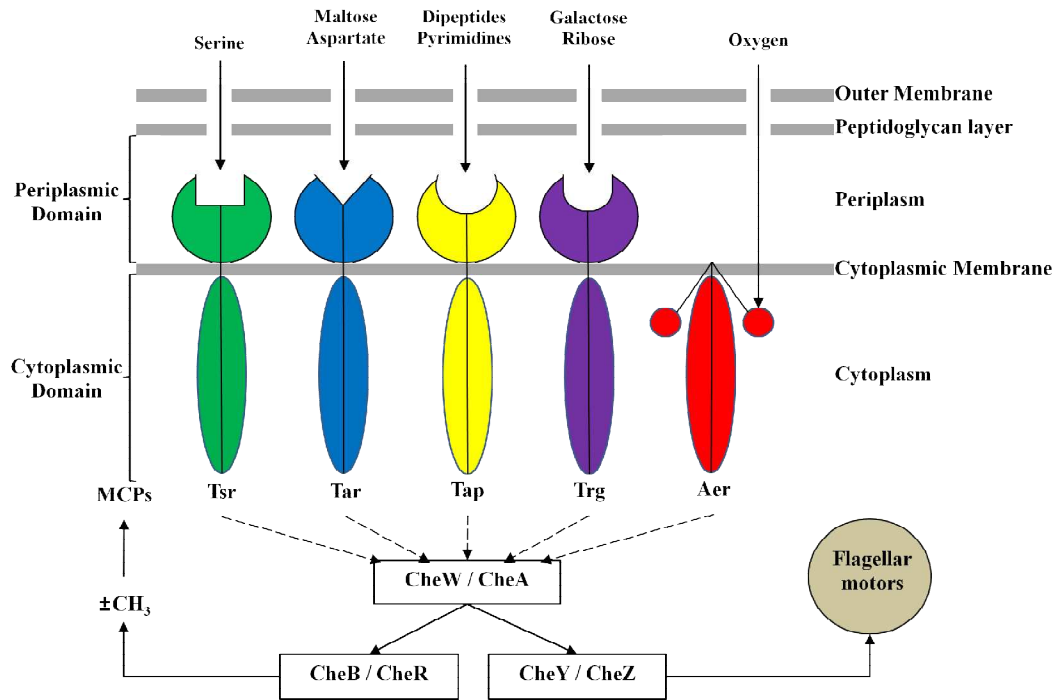


Fig. 2.1. Signaling components and circuit logic of *E. coli*. Five *E. coli* chemoreceptors Tsr, Tar, Tap, Trg, and Aer are shown along with their chemoeffector ligands serine, maltose/ aspartate, dipeptides/pyrimidines, galactose/ribose, and oxygen respectively. All five receptors employ a common set of cytoplasmic signaling proteins, CheW and CheA, which interact with cytoplasmic domain of chemoreceptor to form stable ternary complexes that generate stimulus signals in the form of phosphoryl groups. CheY transmits those signals to the flagellar motors, CheZ controls their lifetime; CheR (methyltransferase) and CheB (methyl-esterase) regulate MCP methylation state.

2.1.2 Methods for studying bacterial chemotaxis

There are several established qualitative and quantitative techniques for assaying bacterial chemotaxis as reviewed by Englert et al. (14). The most commonly used techniques are highlighted below.

2.1.2.1 Swim and swarm plate assays

Chemotaxis of bacteria toward a chemoeffector that can be metabolized can be measured using agar plate assays (14). For swim plate assays, motility medium containing low agar concentrations (0.25 to 0.4%) are used (14). The bacteria move in the aqueous channels inside the agar, and these channels are large enough for them swim through. As the colony grows, it metabolizes any attractants it can which causes the formation of a spatial concentration gradient in the agar. As a result, the cells migrate outward towards higher concentrations (14). By measuring the size of the sharp ring formed by cells at the edge of the steepest gradient, the strength of attraction can be measured (14).

A variation on the swim plate method is the swarm assay. Motility medium containing higher concentrations of agar (0.5% to 0.7%) are used, and the cells swim through the aqueous layer that forms on the agar surface (35). Swarming cells typically produce more, longer flagella (35). Both swimming and swarming assays cannot be used for measuring repellent taxis, and chemotaxis toward chemoeffectors that cannot be metabolized by the cells (14).

2.1.2.2 Capillary assays

This assay was developed by Adler et al. (29) and is widely used for investigating chemotaxis. Chambers on the order of 1 cm² made from plastic o-rings with a 60° cut and 1 mm in height are loaded with a suspension of highly motile bacteria in chemotaxis buffer to create a pond (**Fig. 2.2**). A 1 mm capillary is sealed at one end and filled with several µl of an attractant at the desired concentration at the other. The capillary is then inserted into the pond and incubated at the desired temperature for 45 minutes. The chemoeffector in the capillary will diffuse out into the chamber creating a gradient that can be sensed by the bacteria around the opening of the capillary. The bacteria will migrate into the capillary if the chemoeffector is an attractant. The capillary is then removed, and the contents placed into dilution buffer. Dilutions are plated on nutrient agar and colony counts enumerated, which allow the number of cells entering the capillary to be calculated. These numbers can then be used to compare the chemotactic response and strength of response to various compounds. Although the capillary assay can be used for measuring repellent taxis, the results are not nearly as sensitive as those for attractants.

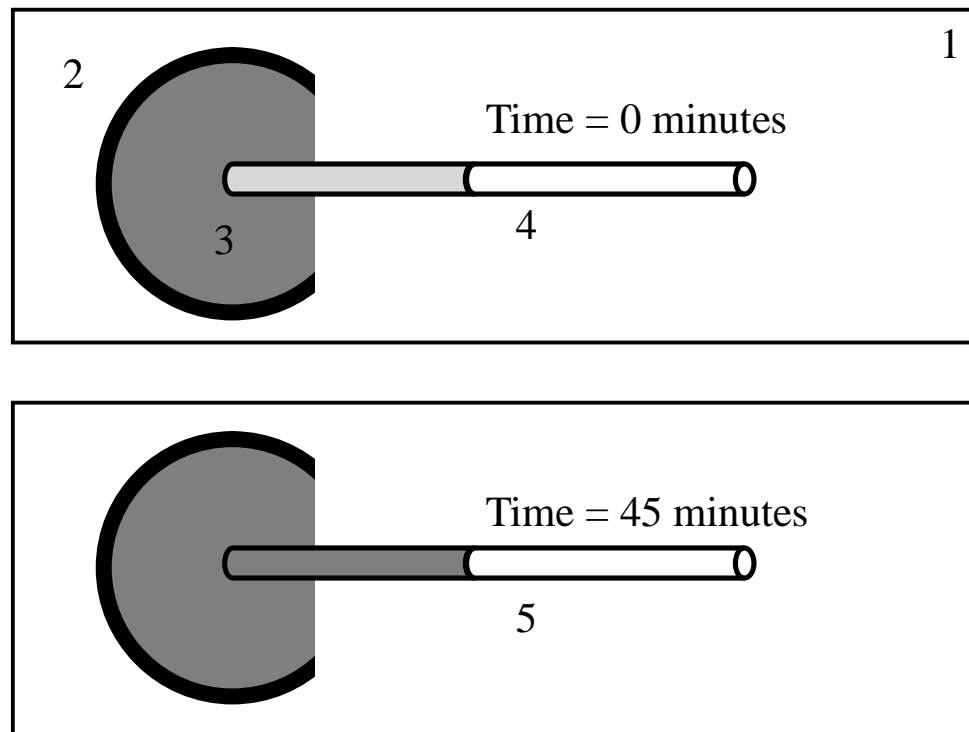


Fig. 2.2. Experimental setup for the capillary assay.

1. Glass slide
2. Plastic o-rings cut at one end
3. Ponds containing highly motile cells
4. Capillary tube containing chemoeffector
5. Capillary tube with cells along with the chemoeffector

2.2 Stress hormones - norepinephrine (NE)

NE is a catecholamine neurotransmitter (stress hormone) that is normally produced in the GI tract through the enteric nervous system (36, 37) and is important in GI-tract infections. The concentration of NE increases during early sepsis (38), and NE has been shown to stimulate the growth of several Gram-negative and -positive bacteria that are present in the intestinal lumen (39). Alverdy and co-workers (6) have correlated the increased NE in the luminal contents of mice after a 30% hepatectomy to increased expression of the *P. aeruginosa* virulence determinant PA-I lectin and gut-derived sepsis. The NE released in the GI tract during stress has also been reported to influence the virulence and infection of other GI tract pathogens. Bansal *et al.* (9) and others (40, 41) have shown that NE increases EHEC O157:H7 attachment and colonization to epithelial cells and colonic mucosa, respectively. NE also enhances the growth, motility, and invasiveness of *Campylobacter jejunii* (42), the expression of the K99 pilus adhesin virulence-related factor in ETEC (43), EHEC O157:H7 virulence gene expression (9, 44), and EHEC O157:H7 chemotaxis, motility, and biofilm formation (9).

CHAPTER III

INVESTIGATING CHEMOTAXIS TOWARDS NOREPINEPHRINE USING MICROFLUIDIC PLUG ASSAY

3.1 Overview

Norepinephrine, one of the eukaryotic signaling molecules present in the GI tract, is considered to be important in the context of GI tract infections. NE is produced at very high concentration in the intestine under post traumatic stress and is known to increase bacterial virulence and infection, and has been shown to be a potent attractant for GI tract pathogens. Bacteria can sense and respond to such attractants using cell surface receptors. By comparing the current concentration of a specific molecule and the concentration detected a few seconds earlier, bacteria determine the net direction of movement to approach attractants or to avoid repellents. This movement is potentially important in the initiation of pathogenic bacteria attachment and infection.. In this study we use a plug-flow assay to show that *E. coli* sense NE through the chemoreceptor Tsr, and elucidate the molecular mechanisms underlying the chemotaxis of these pathogen towards NE. Through these results we expect to further our understanding of bacterial chemotaxis and its role in bacterial colonization and infection of the human GI tract.

3.2 Introduction

Escherichia coli is a serious cause of gastroenteritis (23). This bacterium is noteworthy because a few, but significant, number of infected people develop the

haemolytic uraemic syndrome, which is the most frequent cause of acute renal failure in children in the Americas and Europe (23). Many infections of *E. coli* could be prevented by the more effective application of evidence-based methods, which is especially important because once an infection has been established, no therapeutic interventions are available to lessen the risk of the development of the haemolytic uraemic syndrome (23). Moreover, a recent study (9) has shown that *E. coli* can utilize eukaryotic and prokaryotic signals, such as epinephrine (45) and NE, to “sense” and detect the appropriate location (i.e., large intestine) prior to colonization. Sensing of EPI and NE by *E. coli* could be the first step in a sequence of events leading to infection, as phenotypes such as adherence and colonization, which are downstream of chemotactic recognition and important for infection, are also up-regulated by EPI and NE (9). Indeed, catecholamines have been shown to increase the adherence of *E. coli* to intestinal mucosa in different in vivo models of infection (40, 41). The recognition of EPI and NE by *E. coli* are also in agreement with other reports on the regulation by EPI and NE of the expression of virulence genes and infection (46-48) and, together, suggest an important role for these molecules in *E. coli* infections. Although previous studies have shown that NE increases virulence and pathogenicity, the mechanisms through which NE impacts virulence of different bacteria are not fully understood.

The goal of this work was to investigate the sensing mechanism of NE by *E. coli*. From the lower concentration of 50 μ M, representative of NE levels present in the GI tract during homeostasis that has been used in recent studies (49) to investigate the effect

of hormones on GI tract pathogens, to the higher concentration (200 μ M) was used as representative of supra-physiological levels of NE likely to be encountered in the GI tract during catabolic stress was used in experiments. To our knowledge, this is the first report investigating the sensing mechanism NE by *E. coli*.

3.3 Materials and methods

3.3.1 Bacterial strains, culture conditions, and growth media

A series of knockouts for the four MCP-encoding genes of the *E. coli* strain RP437 was used in this study. For unity of nomenclature, RP437 strain was called CV1, and the mutant series uses the CV designation. All combinations of single (four mutants), double (six mutants), triple (four mutants), and quadruple (one mutant) knockouts were made in the laboratory of Prof. Michael Manson, Department of Biology, Texas A&M University, to create the CV1 through CV16 strains. In this study, only CV1 - CV10, CV12, and CV13 was used. Details on the chemoreceptors present in each of these strains is given in **Table 3.1**. Tryptone broth (TB; 10 g/L tryptone and 8 g/L NaCl) was used to grow all strains. *L*-serine and *L*-aspartic acid were obtained from Fisher Scientific (Fair Lawn, NJ), and the L-(-)-Borepinephrine-(+)-bitartrate (NE) was obtained from Calbiochem (La Jolla, CA).

The bacterial strains and plasmids used in this study are listed in **Table 3.1** All strains were transformed with plasmid pCM18 that encodes for green fluorescent protein

(GFP). Different CV strains with plasmid pCM18 were grown overnight at 32°C in tryptone broth containing 150 µg/ml erythromycin.

Table 3.1 Characteristics of the strains and plasmids used

Strain or plasmid	Genotype	Resistance ^a
<i>Escherichia coli</i> strains		
CV1	Chemotaxis wild type (same as RP437)	Str
CV2	CV1 Δtap	Str
CV3	CV1 Δtrg	Str
CV4	CV1 Δtar	Str
CV5	CV1 Δtsr	Str
CV6	CV1 $\Delta trg-tap$	Str
CV7	CV1 $\Delta tar-tap$	Str
CV8	CV1 $\Delta tar-trg$	Str
CV9	CV1 $\Delta tsr-tap$	Str
CV10	CV1 $\Delta tsr-trg$	Str
CV12	CV1 $\Delta tar-trg-tap$	Str
CV13	CV1 $\Delta tsr-trg-tap$	Str
TG1/pDS-Red Express	Wild type; dead-cell control	Amp
Plasmids		
pCM18	GFP-expressing vector	Erm
pDS-RedExpress	RFP-expressing vector	Amp

a. Str, streptomycin; Amp, ampicillin; Erm, erythromycin

3.3.2 Fabrication of the μ Plug device

Devices were fabricated using standard soft lithography procedures (50). Device drawings were made in AutoCAD or equivalent CAD software and a photolithography mask created from the CAD file using a high-resolution (>3000 dpi) printer (Advanced Reproductions, North Andover, MA). The device was created photolithographically using silicon wafer and SU-8 negative photoresist (Microchem Corp, MA). A 10:1 mixture of PDMS prepolymer and curing agent was prepared and cured against the Si master. After curing for 4h at 85 °C, the PDMS replica were removed from the master and the cell-inlet and agarose-inlet ports were made using a needle. The device was then oxidized in an oxygen plasma etcher (100 mTorr, 100 W, 40 sec) with a glass slide for 40 s. Bringing the oxidized PDMS and glass slide into conformal contact resulted in an irreversible seal between the PDMS mold and the glass slide.

The μ Plug assay (14, 51) is an improved version of the well-established plug-in-pond assay (32). It consists of a 15 X 15 mm square chamber with a height of ~75 μ m. Agarose mixed with chemoeffector is introduced through a 1.5 mm diameter hole in the middle of the chamber. Two additional holes are punched with a blunt 19-gauge needle along the diagonal, one of which to introduce cells into the chamber, and the other to provide a vent as shown in **Fig. 3.1**. The gradient of chemoeffectors are formed by diffusion out of the plug. Depending on what the orientation of the cells are to the chemoeffector inside the plug, cells either move away (if repellent) or towards the plug (if attractant). (case of attractant is shown in cartoon in **Fig. 3.1**)

While the μ Plug assay results provide whether the chemoeffector placed in the plug is an attractant or a repellent, it does not facilitate quantification of the extent of response towards or away from the chemoeffector. Furthermore, this assay is also prone to false positives as some cells may attach to the plug when being inserted into the chamber. There is also some variability in the number of cells (and hence, the apparent magnitude of response) because the cell density used in the assay cannot be rigorously controlled and more cells are typically observed near the cell port, with the density decreasing at distances farther from it. Although the results shown here are reproducible, it is always desirable to confirm the μ Plug results with additional assays such as the capillary assay.

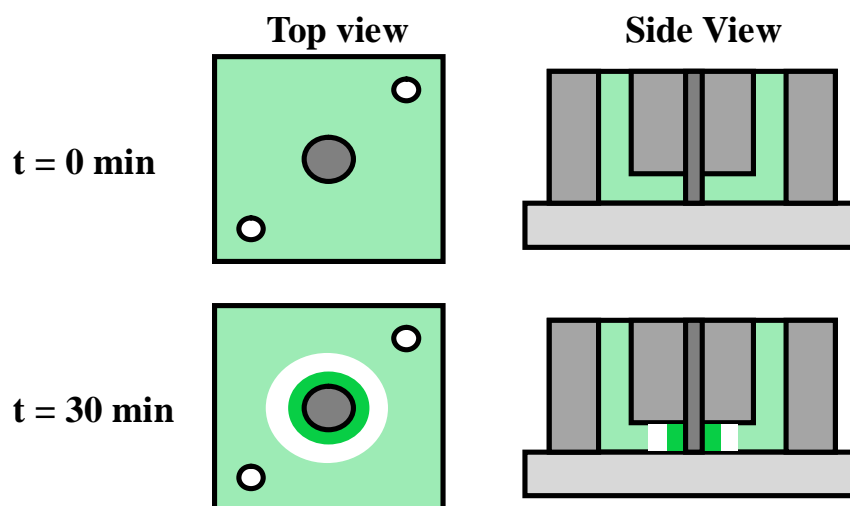


Figure 3.1 Schematic of the modified μ Plug assay. Both top and side views of the chamber are shown. GFP-labeled bacteria suspended in CB were introduced at the bacteria inlet, and the air outlet allowed escape of air. The agarose plug contained CB plus *L*-serine or NE at the desired concentration. The plug was visualized by addition of 5% bromophenol blue to provide optical contrast. Gradients in the bacterial suspension form by diffusion of attractant out of the plug. Gradients develop rapidly and are relatively steep. The cartoon shows the distribution of GFP-labeled cells when they are first introduced ($t = 0$ min) and at the end of the experiment ($t = 30$ min).

3.3.3 Growth of bacteria for the μ Plug assay

Bacteria were prepared for chemotaxis assays as described earlier in our prior work (14). Briefly, The overnight culture was diluted to a turbidity of 0.05 at 600nm in 25 ml of TB without erythromycin and then grown to mid-logarithmic phase (turbidity of ~ 0.5 at 600 nm) at 32°C. Prior to preparing cells for the assay, bacterial motility was visually examined using phase-contrast microscopy to ensure robust motility and normal run-tumble swimming behavior. Cells were centrifuged at 400 x *g* for 10 minutes, and the aliquot was discarded. Chemotaxis buffer (CB; phosphate-buffered saline, 0.1 mM EDTA, 0.01 mM L-methionine, and 10 mM D,L-lactate) was added to the cells so that the concentration of the cells was twice of the density before centrifugation.

Cells were mixed with an approximately equal number of dead TG1 cells that had expressed red fluorescent protein (RFP) from plasmid pDS-Red Express (Clonetech, Mountain View, CA). The dead RFP-containing cells served as a control to visualize any mixing due to turbulence within the microfluidic chamber.

For experiments involving priming of cells with NE, cells were exposed to a 2 μ M of NE 30 minutes prior to harvest of cells. Control cells were primed with CB. A fresh stock of NE was used for all experiments.

3.3.4 μ Plug assay

The agarose plug was made by melting 20 mg low-melting-temperature agarose in 950 μ L of CB and 50 μ L of 5% bromophenol blue solution (to provide optical contrast)

at 70°C. The temperature of the agarose was reduced to 55°C, and chemoeffector was added to the final concentration desired and thoroughly mixed. An 8 μ L aliquot of the agarose mixture was introduced into the μ Plug device via the center hole. The device was allowed to sit for 5 min to cool to room temperature. The mixture of GFP-expressing (live) and RFP-expressing (dead) cells was introduced gently via one of the corner holes until the chamber was full, taking care to avoid air bubbles. Green and red fluorescent images of the cells around the plug were taken immediately after the device was placed on the microscope stage and after 30 min at room temperature (\sim 23°C). The uniform distribution of red cells was used to ensure that no bulk flow had occurred.

3.3.5 Capillary assays

Capillary assays were performed as previously described (29) except that plastic gaskets of the proper diameter and thickness were used to create the chamber, or “pond.”. Capillaries contained either CB alone or CB with the indicated concentration of *L*-serine, *L*-aspartic acid, or NE. The assay was run for 45 min at 32°C, and the number of cells entering the capillary was determined by plating dilutions of the capillary contents on LB agar containing 50 mg/mL streptomycin and counting colonies after 24 h incubation at 37°C. While this assay is simple, provides reproducible results, and the diffused gradients of a capillary has been mathematically characterized (52, 53), the sensitivity of this assay is somewhat limited because of the relatively low numbers of cells that are

exposed to a detectable concentration gradient, and the concentration gradient becomes shallower over time until it finally disappears.

3.3.6 Preparation of bacteria for the capillary assay

The protocol for growth of bacteria was identical to that for the μ plug assay, except that CB was added so that the concentration of the cells was decreased 10-fold than before the centrifugation. For experiments with higher concentration of cells in the pond, the cell density was increased two-fold. In case priming was involved, 2 μ M of NE were added to the cells to be primed 30 minutes prior to harvest of cells.

3.4 Results

3.4.1 Response of wild-type cells

The responses of *E. coli* strain CV1 (RP437) and its isogenic *tsr* mutant derivatives are shown in **Fig. 3.2**. In the absence of NE in the plug, CV1 cells distributed themselves randomly (**Fig. 3.2(a)**). However, when CV1 cells were exposed to plugs containing 200 μ M *L*-serine or 200 μ M NE, they exhibited strong attractant responses, as shown by the accumulation of bacteria at the agarose plug-liquid interface (**Fig. 3.2(b)** and **(c)**) confirming that NE is indeed an attractant to the cells.

3.4.2 Response of cells lacking different chemoreceptors

Strains CV2 (CV1 Δtap), CV3 (CV1 Δtrg), and CV4 (CV1 Δtar) responded to *L*-serine and gave a positive response to NE as well (**Fig. 3.3**). However, strain CV5 (CV1 Δtsr) did not respond to either *L*-serine or NE. These results led to the conclusion that Tsr, which is the receptor required for taxis towards *L*-serine, is involved in NE sensing.

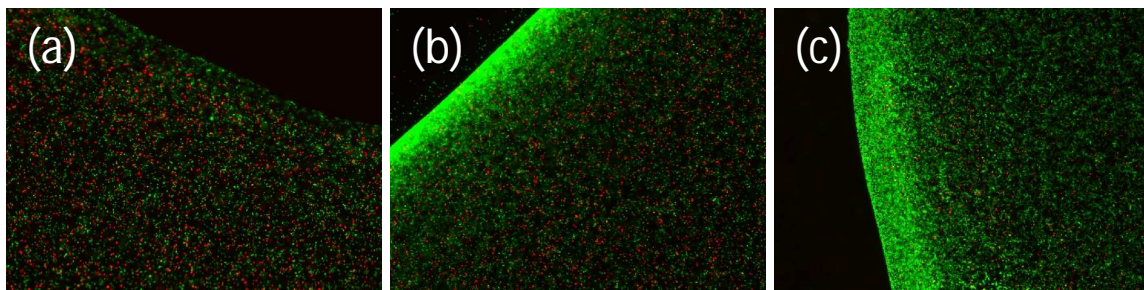


Figure 3.2 Chemotactic response of CV1(RP 437) (a) Wild-type (CV1) cells in the absence of any attractant in the plug. (b) Distribution of wild-type (CV1) cells with 200 μ M *L*-serine in the plug. (c) wild-type (CV1) cells with 200 μ M NE in the plug.

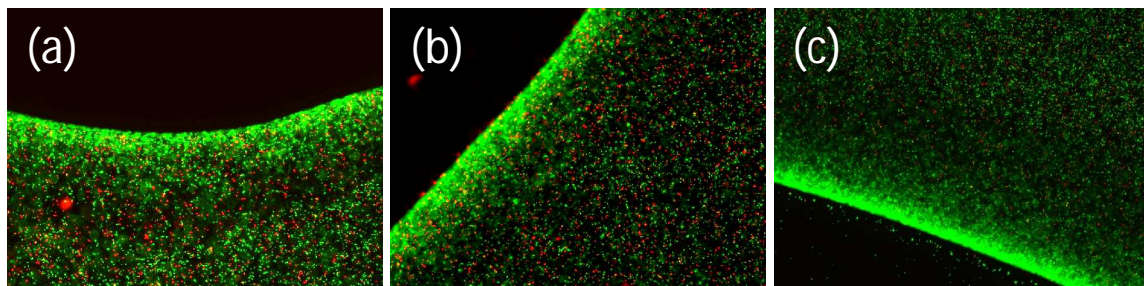


Figure 3.3 Chemotactic responses to NE in the μ Plug assays. Figures show the results of (a) Δtap (CV2), (b) Δtrg (CV3), (c) Δtar (CV4) cells with 200 μ M NE in the plug.

To confirm that the Tsr is the receptor solely responsible for the detection of NE, the mutant with only Tsr (CV 12) was used. Strain CV12 (CV1 $\Delta tar-tap-trg$), which has Tsr as its only functional receptor (other than Aer), responded to *L*-serine (data not shown) but not NE (**Fig. 3.4(a)**), showing that Tsr receptor by itself is not sufficient for NE chemotaxis in *E. coli*.

We then determined if a second receptor is required for chemotaxis towards NE. This experiment was done using 5 different strains with double knockouts - strains CV6 (CV1 $\Delta trg-tap$), CV7 (CV1 $\Delta tar-tap$), CV8 (CV1 $\Delta tar-tag$), CV9 (CV1 $\Delta tsr-tap$), and CV10 (CV1 $\Delta tsr-trg$). **Fig. 3.4(b)-(f)** shows that strains with at least one additional receptor other than Tsr shows attraction to NE. Of the 5 strains with double knockouts, only strains CV6, CV7, and CV8, which all have Tsr and one other receptor (Tar, Trg, and Tap, respectively) are attracted to NE.

3.4.3 Effect of priming

It is well established that pre-exposure to specific ligands prior to testing their chemotactic response increases the extent of chemotaxis observed (54, 55). For example, maltose chemotaxis requires that cells be exposed to maltose, as this leads to expression of the maltose binding protein that binds with the ligand and the Tar chemoreceptor. Therefore, we tested the effect of priming cells with NE prior to NE chemotaxis (56).

Exposing the wild-type CV1 cells to NE (**Fig. 3.5(a) and (b)**) and *L*-serine (data not shown) did not result in a significant increase in the extent of chemotaxis. However, priming with NE prior to chemotaxis led to a marginal chemotaxis response in strain CV12. However, the extent of attraction was weaker than that observed with CV1.

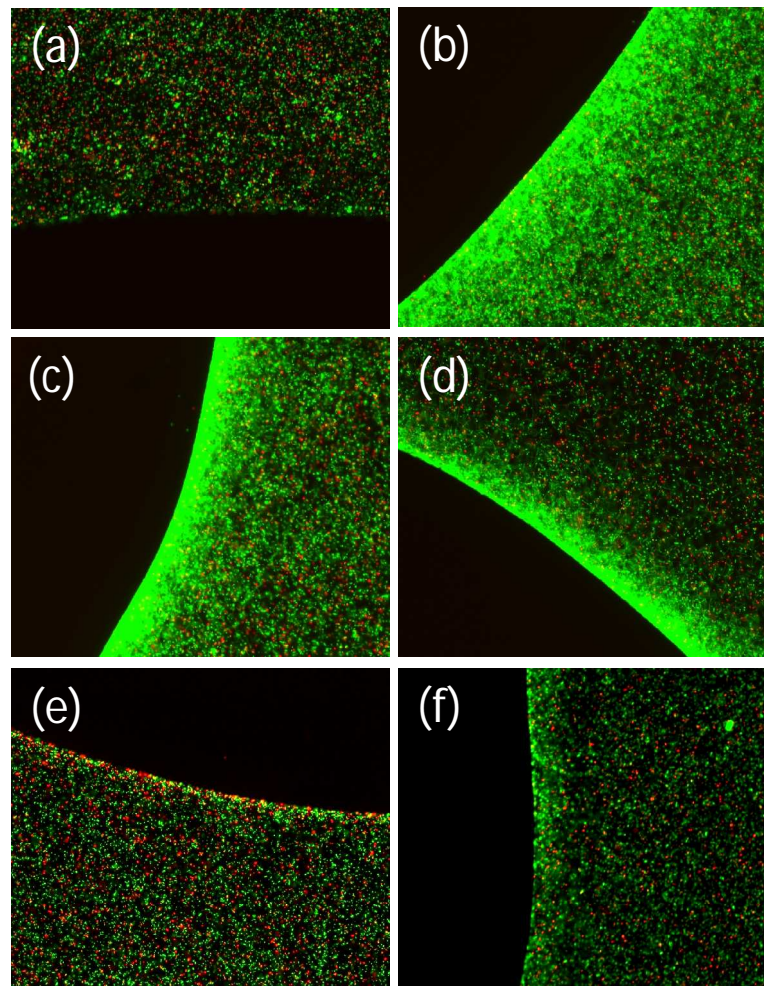


Fig. 3.4. Chemotactic responses of triple-knockout strain CV5 and double-knockout strains to NE in the μ Plug assays. Figures show the results of (a) $\Delta tar-tap-trg$ (CV12) (b) $\Delta trg-tap$ (CV6), (c) $\Delta tar-tap$ (CV7), (d) $\Delta tar-trg$ (CV8), (e) $\Delta tsr-tap$ (CV9), and (f) $\Delta tsr-trg$ CV10 cells with 200 μ M NE in the plug.

3.4.4 Capillary assay confirmation of plug assay results

Capillary assays (29) were used to quantitatively determine the strength of the NE chemotaxis response. As expected from the μ Plug assay results, CV1 cells accumulated in capillaries containing *L*-serine or NE (**Fig. 3.6**), and CV5 (Δ t_{sr}) cells did not accumulate in capillaries containing either *L*-serine or NE. Thus, the conclusions from the \square Plug assay on the requirement of T_{sr} for NE chemotaxis were confirmed.

From the plot of the number of accumulated cells in the capillaries with chemoeffectors, *L*-serine and 500 μ M and 5000 μ M NE, we can see that there is no significant increase in accumulation of CV1 and CV12 cells in capillaries containing NE, either with priming or without priming (**Fig. 3.6(a)**). This lack of accumulation confirms the conclusions from the second set of the μ plug assay on the hypothesis of requirement of a second receptor. However, when the experiment was carried out with approximately 10 times more cells in the capillary than the normal protocol, significant accumulation of CV1 and CV12 cells in NE capillaries are observed (**Fig. 3.6(b)**). The effect of priming can also be seen in **Fig. 3.6(b)**, where almost 2-fold increase in the number of cells is observed with the normal cell density and up to more than 3-fold increase with the NE sample in case of high cell density protocol.

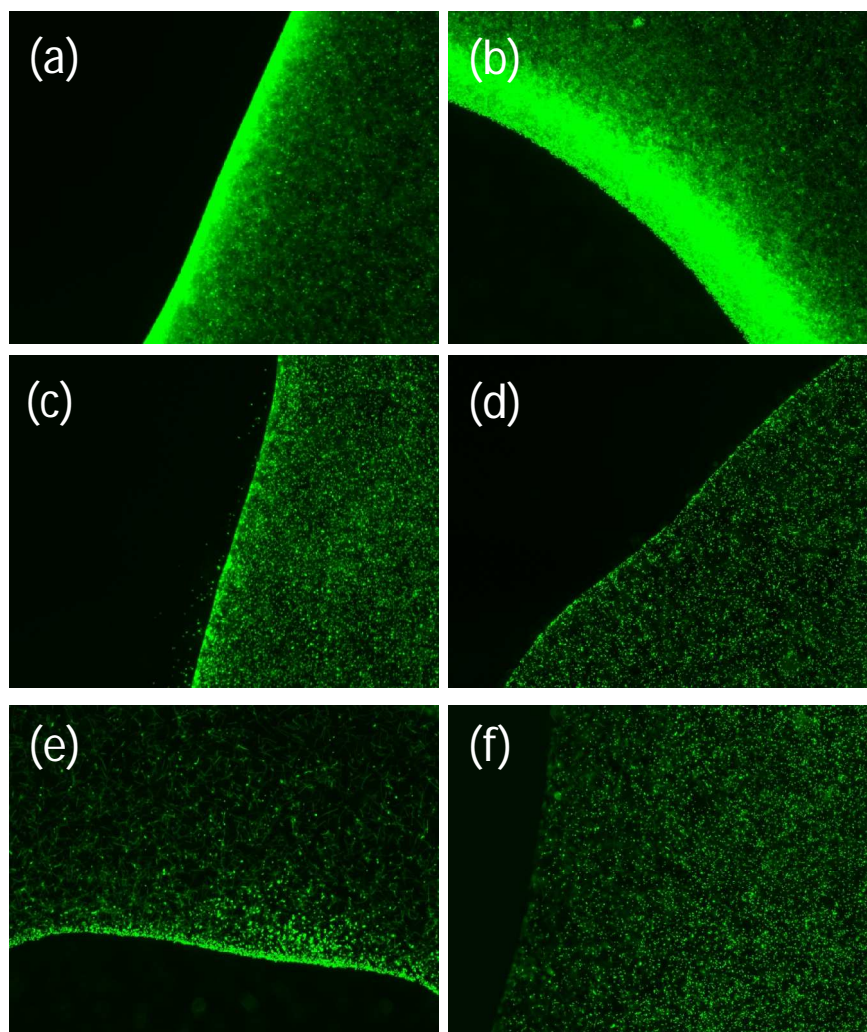


Fig. 3.5. Effect of priming on chemotactic responses of CV1, CV5, and CV12 to NE in the μ Plug assays. Figures show the results of (a) CV1 with priming (b) CV1 without priming, (c) CV5 with priming, (d) CV5 without priming, (e) CV12 with priming, and (f) CV12 with priming with 200 μ M NE in the plug.

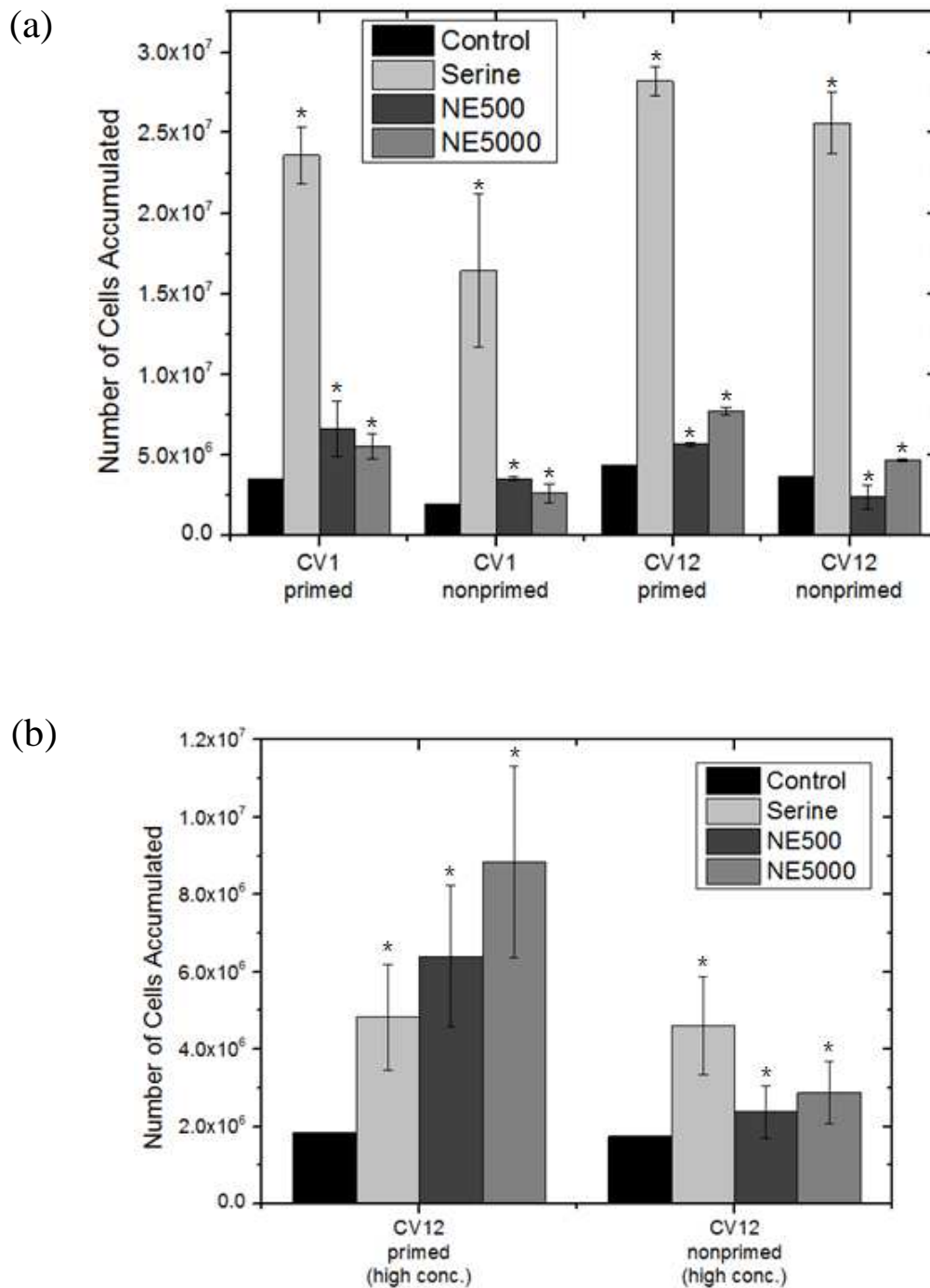


Figure 3.6 Comparison of the responses of cells to *L*-serine and NE in the capillary assay. (a) Number of primed and non-primed CV1 and CV12 cells, and (b) primed and non-primed CV12 in experiment carried out from higher number of cells, exposed to *L*-serine, 500 μ M, and 5000 μ M norepinephrine. (* indicate statistical significance with respect to the control determined using the Student t-test at $p < 0.05$.)

CHAPTER IV

DISCUSSION, SUMMARY, AND CONCLUSIONS

4.1 Discussion

Our data suggest that of the four MCP's of *E. coli*, Tsr has a major role in detection of NE. Since NE is derived from the amino acid tyrosine, may interact with a site that is identical to, or at least overlaps with, the known serine-binding site. Tsr analogues have been reported in many bacterial species including enteric pathogens (57), and it is possible that the response to NE is mediated through Tsr in the pathogenic bacteria as well.

Sensing of NE by *E. coli* could be the first step in a sequence of events leading to infection, as phenotypes such as adherence and colonization, which are downstream of chemotactic recognition and important for infection, are also up-regulated by NE. Indeed, catecholamines have been shown to increase the adherence of *E. coli* to intestinal mucosa in different in vivo models of infection (40, 41). NE has been previously reported to induce bacterial growth (58), and there are reports in the literature that imply that NE might function as a siderophore (58). NE has been implicated as inducing expression of enterobactin and iron uptake in *E. coli*, suggesting that this is the mechanism involved in growth induction (59). However, the role of NE in bacterial pathogenesis seems to be more complex, because several reports suggested that this signal stimulates the growth of low cell density *E. coli* and induces the production of an autoinducer (AI) which, in turn, promotes *E. coli* growth (59).

From the observation in this study, the recognition of NE by *E. coli* seems to require the Tsr receptor, but this receptor by itself is not sufficient. This is supported by the μ plug-assay results where a strain expressing Tsr and any other chemoreceptor responds to NE but a strain expressing only Tsr does not do so. It is not clear at this point if the second receptor is required for binding to NE or if it merely provides a critical density of chemoreceptors (i.e., a certain number of receptors in the receptor patch) in the receptor patch of *E. coli* (60, 61). The strains with knockout of receptor(s) used in this study have receptor patch with fewer receptors compared to the wild-type RP437 strain. For example, the size of the receptor patch for the CV12 strains is only a quarter of the size in CV1 strains (62). Thus, it is possible that that strain CV12 lacks a critical density of chemoreceptors in the receptor patch required for efficient chemotaxis.

Cells are able to sense and respond to various external stimuli. To extend the working range of their sensory pathways, biochemical mechanisms allow for adaptation to persistent stimulation, resulting in only a transient response. The dynamics of adaptation are important as they often represent the cellular memory of previous environmental conditions, directly affecting cellular behavior (54, 63-65). Moreover, for some cases of chemotaxis, the chemoeffector does not directly bind to the respective receptor but interacts with the receptor through a binding protein that is expressed only in the presence of the chemoeffector. For example, maltose binding protein is expressed when cells are exposed to maltose, and the maltose-maltose binding protein binds to the aspartate receptor Tar (56). Similar observations have been made for the chemotactic response of galactose and ribose, where binding of the galactose-binding protein (66)

and ribose-binding protein (67) with Trg is required for chemotaxis to galactose and ribose, respectively. Recently, our lab demonstrated that the autoinducer-2 (AI-2) quorum sensing molecule binds to the periplasmic LsrB for chemotaxis but it is not clear if LsrB expression is induced upon exposure to AI-2. (68). Our data showing that priming cells with NE increases the chemotactic response suggest that pre-incubation may lead to the induction of any co-receptor(s) required for chemotaxis. While the effect of priming was not fully observable in the μ plug assay, capillary assays confirmed that around 2-fold increase in attraction has occurred in case of primed strains. Moreover, when the experiment was carried out with a higher cell density, the effect of priming was further magnified, which suggest that the effect of priming could be a cell-density dependent metabolic response.

For all the experiments in this work, cells were grown in tryptone broth (69). Compared to Luria Bertani medium (LB), TB lacks yeast extract which contains nucleic acids, lipids and other nutrients which are needed for bacterial growth (70). Also, prior to the assay, the bacteria are suspended in chemotaxis buffer (CB), which contains only 10mM of *D, L*-lactate as the energy source. Given that the growth media and the buffer used provide the cells only minimal nutrients, it is possible that cell are constantly seeking other energy sources. Based on studies showing that aromatic compounds can be utilized by *E. coli* as carbon and/or nitrogen sources (71), it is intriguing to speculate that the increased response observed with NE priming could be due to a response to a molecule that is produced during the biodegradation of NE by *E. coli*. This is also

consistent with studies showing that NE potentiates bacterial growth and induces expression of virulence determinants in *E. coli* (15, 72).

4.2 Summary and conclusions

We have shown that Tsr receptor is necessary for chemotaxis of NE for *E. coli* RP437, and attraction of *E. coli* towards NE may require an additional receptor. Results from the priming experiments suggest that exposure to NE may result in the de novo expression of co-receptor(s) that are crucial to chemotaxis towards NE. The requirement for high cell density also suggests the possibility that NE per se may not be an attractant for *E. coli*, but could be a precursor that is modified into a chemoattractant by cells (i.e., conceptually similar to the idea proposed by Goldberg et al, (73)).

4.3 Future work

The hypothesis that increasing number of receptors of even a single Tsr receptor leads to enhanced chemotactic response to NE needs to be investigated further. Specifically, we will need to determine if a specific receptor (e.g., Tar) or binding site is required for chemotaxis towards NE or whether the attenuated response with the single receptor is due to a lack of sufficient receptor density in the receptor patch. This can be tested by using CV16 (lacks all four MCPs) and introducing plasmid pCAN24-Tsr into it. Since the expression of Tsr in this plasmid can be induced, by using different concentrations of IPTG, we can achieve different Tsr levels in CV16 cells. By

correlating the extent of chemotaxis to the level of Tsr induction, we can determine if additional receptor(s) are required.

The plug assay used in this work provides a qualitative determination of chemotaxis but does not allow comparison of responses. For example, what is the extent of response with different levels of forced over-expression of Tsr? This can be addressed by using a microfluidic chemotaxis flow system developed in our lab (**Fig 4.1(a)**) (14). Such a model can be used to determine the optimum concentration needed for chemotaxis. This device will also allow us to study chemotaxis within the GI tract more effectively by simulating different flow patterns and profiles.

Most importantly, the effect of priming should be investigated further to elucidate the mechanism underlying *E. coli* chemotaxis. The requirement for de novo synthesis can be investigated by adding a protein synthesis inhibitor and determining the extent of chemotaxis (**Fig 4.1(b)**). Similarly, we can also utilize the genome information available at the KEGG database (74) to identify potential molecules that can be generated from NE and the pathways that are involved.

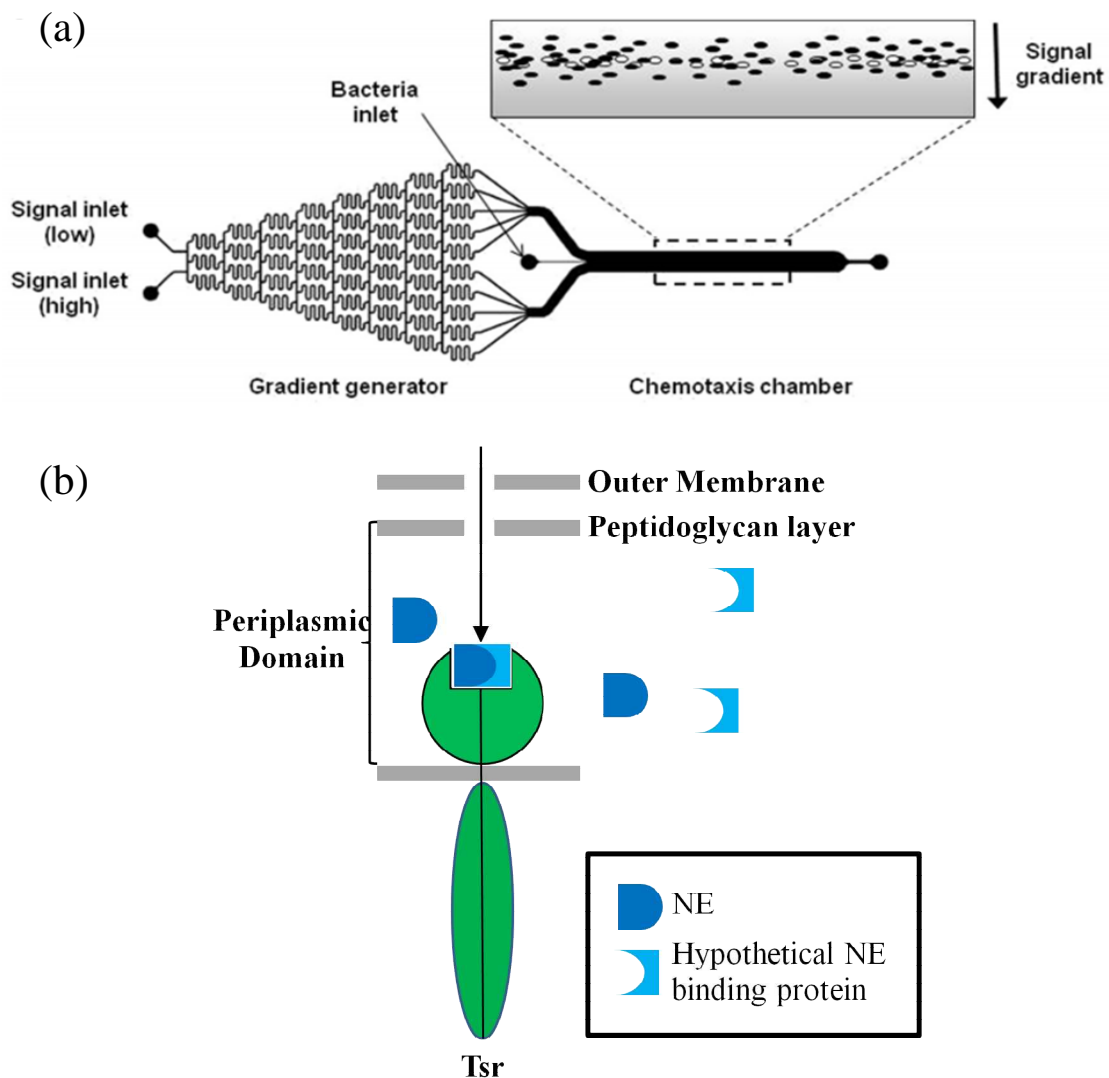


Fig. 4.1. Future work. (a) Schematic representation of the microfluidic device. The device consists of a gradient-mixing module and a chemotaxis observation module. The inset schematically depicts a gradient of a repellent molecule (gray) and bacteria migrating in response to it. (51) (b) A hypothetical NE binding periplasmic protein that may bind to NE and interact with the Tsr receptor.

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